Spectrophotometric Determination of Cholesterol and Triterpene Alcohols in Wool Wax

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Colorimetric methods previously used for the determination of cholesterol and triterpene alcohols in wool wax did not take into account the overlapping of the absorption bands of the colored derivatives. Other disadvantages were the necessity of keeping the temperatures of the test solutions below 12° C. and the fact that, at the wave lengths selected, a sample of suitable concentration for determination of cholesterol was much too concentrated to be used for the triterpene alcohols. The procedure was modified in the following ways. The addition of 1,4-dioxane to the reagent reduces the rate of color development and permits operation at room temperature. By substituting a spectrophotometer for the colorimeter and determining the triterpenes at the secondary maximum at 550 mμ and cholesterol at 630 mμ, a single solution can be used for both. Corrections are made for overlapping absorption bands in both cases. Various modifications make the method applicable to the unsaponified wool wax esters, to the mixture of free alcohols obtained by saponification of wool wax, or to precipitated cholesterol digitonide.

THE so-called unsaponifiable or alcohol fraction of wool wax consists of approximately 30% steroids, 25% triterpenes, 15% acyclic primary monohydric alcohols, 5% acyclic 1,2-diols, and about 25% of uncharacterized material. Apparently the acyclic alcohols are predominantly branched chain compounds. In the steroid portion cholesterol predominates, but it is accompanied by β-cholestanol (dihydrocholesterol) in quantities ranging from 2 to 6% of the total alcohol fraction and smaller proportions of 7-ketocholesterol (*2*). The triterpene alcohols present include the predominant lanosterol (lanostadienol), smaller quantities of agnosterol (the corresponding trienol), dihydrolanosterol, dihydroagnosterol, and possibly other similar substances as yet unidentified. The relative proportions of these various compounds have not been definitely determined (*10*). A recent publication by Ruzicka *et al.* (*13*) states that these triterpene alcohols are closely related to the steroids. Lanosterol, for example, they consider to be 4,4,14-trimethylzymosterol. Six dextrorotatory ante-iso alcohols with odd numbered chains ranging from C_{17} to C_{27} and four optically inactive iso alcohols with even numbered chains ranging from C_{20} to C_{26} have been reported to be present. This fraction includes those compounds heretofore reported as cetyl and ceryl alcohols (*8*). The five alkyl-1,2-diols contain even numbers of carbon atoms (C_{18} to C_{24} inclusive), but their properties indicate that their alkyl chains have a single methyl branch (*4*).

Fortunately methods for the determination of cholesterol, the major constituent of this complex mixture, have been developed and can be applied to wool wax. The most common methods are the gravimetric method in which cholesterol is precipitated as the digitonide (*14*) and the Liebermann-Burchard method in which the intensity of the color developed by treatment with acetic anhydride and sulfuric acid is measured photometrically (*1, 7*). The Schoenheimer-Sperry procedure, a combination of these two, is widely used for determining combined and free cholesterol in blood serum and other tissues (*12*).

It has been shown that the colored derivatives of the triterpene alcohols produced by the Liebermann-Burchard reaction, like the similar derivatives of cholesterol, obey the Beer-Lambert law, and a procedure based upon this reaction has been applied to the quantitative determination of cholesterol and the triterpene alcohols in wool wax (*6*). This method does not, however, take into account that the colored derivatives of the triterpene alcohols have appreciable absorption in the region where the cholesterol derivative has its maximum and vice versa (Figure 1), and it provides no correction for this overlapping absorption. The method also has the serious disadvantage that the color must be developed and readings made at 12° C. The difficulty of preventing the reaction from proceeding beyond the point of maximum color intensity while the necessary measurements are being made is obvious. The procedure has been modified in the following ways:

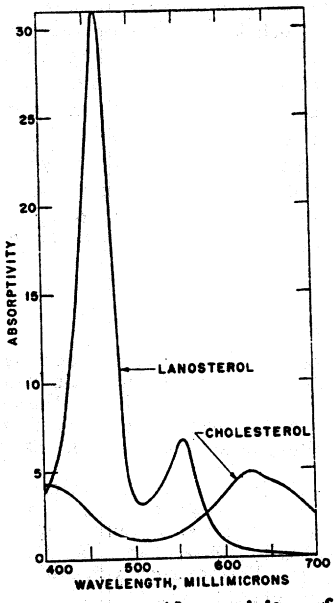

Figure 1. Absorptivity of the Liebermann-Burchard Color Complexes of Lanosterol and Cholesterol

A Beckman Model DU spectrophotometer with a tungsten lamp as the light source is used instead of the Pulfrich photometer and filters used by Lederer and Tchen, and determination of cholesterol is made at 630 mμ and that of the triterpene alcohols at 550 mμ. This secondary maximum in the curve for the triterpene alcohol derivative was chosen because the principal maximum at 458 mμ is so much more intense than that of the cholesterol derivative at 630 mμ (Figure 1) that a single solution suitable for accurate measurements in both regions cannot be prepared.

The addition of 1,4-dioxane to the reagent reduces the rate of development of the color sufficiently to permit the reaction to be carried out at room temperature (20° to 25° C.) and lengthens appreciably the period of maximum color intensity (*11*). The necessary absorptivities were determined by applying the procedures described below to pure samples of cholesterol and lanosterol (Table I).

Table I. Absorptivities[a] of the Liebermann-Burchard Color Complexes

Approximate Concentration, Gram/Liter	Cholesterol[b] a_C, 630 mμ	Cholesterol[b] $a_{C'}$, 550 mμ	Lanosterol[c] a_L, 550 mμ	Lanosterol[c] $a_{L'}$, 630 mμ	Cholesterol Digitonide[d] a_{CD}, 630 mμ
0.025	5.01	1.51	5.48	0.46	4.79
0.050	4.99	1.51	5.53	0.46	4.79
0.075	4.98	1.48	5.49	0.46	..
0.100	5.00	1.51	5.48	0.46	4.79
Av.	5.00	1.50	5.50	0.46	4.79

[a] Absorptivity, $a = \frac{\text{absorbance}}{\text{(total concentration, grams/liter)(layer thickness, cm.)}}$

[b] Purified product isolated from wool wax: m.p. 148.8° C., $[\alpha]_D^{20}$ $CHCl_3$ = −37.6°.

[c] Purified product isolated from wool wax: m.p. 140.1° C., $[\alpha]_D^{20}$ $CHCl_3$ = +56.6°.

[d] Prepared by precipitating with digitonin the pure cholesterol described above. The absorptivity is calculated for the cholesterol equivalent of the digitonide.

In the application of the Liebermann-Burchard reaction to esters of cholesterol it has been shown that not only is the color developed more rapidly than it is with free cholesterol but also the maximum absorbance (optical density) is greater by approximately 20% for equivalent cholesterol content (*5*). This can be obviated by using cholesteryl esters instead of free cholesterol in preparing the standards or by increasing the absorptivity obtained for free cholesterol by 20% in the calculation of the cholesterol content of esters. In either case the result is admittedly only a fairly accurate approximation of cholesterol content when applied to mixtures of free cholesterol and cholesteryl esters. This does not apply to the triterpene alcohols whose esters show no enhancement of color intensity (*6*). It has also been shown that all four of the known triterpene alcohols are equivalent as far as response to the Liebermann-Burchard reaction is concerned (*6*), so that absorption data obtained from pure lanosterol can be used for any or all of the alcohols of the triterpene fraction.

In the case of cholesterol any ketocholesterol which may be present will give the same color reaction as cholesterol, but it is to be remembered that although dihydrocholesterol is precipitated by digitonin (*13*), it contains no unsaturation and cannot give the Liebermann-Burchard color reaction. It has been reported that, although pure lanosterol is strongly precipitated by digitonin, the dihydro terpene alcohols and their mixtures with lanosterol give only slight precipitates (*9*). Precipitation of the triterpene alcohols by digitonin from wool wax alcohol mixtures has never been encountered by the authors.

PROCEDURE

Three distinct methods of procedure have been developed and are described below. Two of these include determination of the triterpene alcohols along with cholesterol. The first of these is applicable to mixtures of wool wax alcohols and the second to the wool wax ester mixture. The third is for the determination of cholesterol only.

Method I. Determination of Cholesterol and Triterpene Alcohols in Mixtures of Free Wool Wax Alcohols. The reagent is prepared by adding (slowly, with shaking) 1 volume of cold (0° C.) concentrated sulfuric acid to 4 volumes of acetic anhydride in a flask immersed in an ice bath. This mixture is then diluted in a similar manner with 2.5 volumes each of glacial acetic acid and purified 1,4-dioxane. After mixing thoroughly the reagent is held at 0° C. until used. It is recommended that the 1,4-dioxane be purified by Fieser's method (*3*). All volumes should be accurately measured. The reagent is relatively unstable and should be rejected if it develops any color or if it is not used the same day it is prepared.

The sample to be analyzed is dissolved in chloroform to a standard volume. For most samples a concentration of 10 mg. in 100 ml. of chloroform solution is satisfactory. A 10-ml. aliquot of this solution is pipetted into a 25-ml. glass-stoppered, graduated cylinder, and 5 ml. of the reagent at 0° C. is added. The stoppered cylinder is then inverted several times to mix the solutions thoroughly and placed in a dark cupboard at room temperature (20° to 25° C.). Color development reaches its maximum after about 30 minutes and remains at approximately that level for about 10 minutes. Transfer of samples to 2.5-cm. absorption tubes with wide neck openings is begun after 25 minutes' standing, and readings are made during the 10-minute interval of maximum color intensity. The absorbance (optical density) is measured at 550 mμ for the triterpene alcohols and at 630 mμ for cholesterol. A blank consisting of 10 ml. of chloroform and 5 ml. of the reagent is used to zero the instrument at each wave length. The measurements are carried out at constant instrument sensitivity so adjusted that the blank solution at 550 and 630 mμ requires slit widths of approximately 0.25 and 0.10 mm., respectively, to bring the instrument into balance.

Method II. Determination of Cholesterol and Triterpene Alcohols in Unsaponified Wool Wax Esters. The procedure is the same as in Method I except that the original chloroform solution contains 20 mg. of sample per 100 ml.; transfer of the samples to absorption tubes is begun after 20 minutes' standing; and readings are made during the subsequent 10-minute period. This is necessary because color development is more rapid with cholesteryl esters than with free cholesterol. There is little if any difference between the rate of color development for triterpene alcohols and their esters (*6*).

Method III. Determination of Cholesterol Only in Wool Wax. By this procedure the total cholesterol can be determined or alternatively, if the saponification step is omitted, free cholesterol only. The results may differ from those obtained in the gravimetric digitonin method if dihydrocholesterol is present, since this compound, which is precipitated by digitonin and weighed as cholesterol in the latter method, does not respond to the Liebermann-Burchard color reaction. Only cholesterol is measured by this procedure. It is necessary to modify the reagent for this method as the digitonide cannot be dissolved in chloroform. In making up the reagent the sulfuric acid and acetic anhydride are mixed as described under Method I, but diluted with 2.5 volumes each of chloroform and 1,4-dioxane. The proper proportion of glacial acetic acid is introduced later as the solvent for the digitonide sample.

Approximately 200 mg. of wool wax is accurately weighed, dissolved in absolute ethanol or, if insufficiently soluble in that solvent, ethanol containing about 20% 1,4-dioxane, and made up to a volume of exactly 100 ml. In an ordinary 15-ml., conical centrifuge tube, 5 ml. of this solution, approximately 0.1 gram of 50% aqueous potassium hydroxide, and 1 drop of 1% alcoholic phenolphthalein solution are thoroughly mixed. The solution is boiled gently for 1 minute, and the tubes are loosely stoppered and placed in a sand bath in an oven at 50° to 60° C. for 3 hours

Table II. Analysis of Test Mixtures for Cholesterol and Lanosterol by Method I

Sample No.	Actual Content, % Cholesterol	Actual Content, % Lanosterol	Found by Analysis, % Cholesterol	Found by Analysis, % Lanosterol
1[a]	40	60	40.6	63.5
2[a]	50	50	48.5	50.6
3[a]	60	40	60.2	40.8
4[b]	56.9	..	57.4	..
5[c]	..	52.9	..	54.3
6[d]	50	20	49.4	21.7

[a] Mixtures of pure cholesterol and pure lanosterol in the proportion indicated.

[b] 180.6 parts by weight of wool wax alcohols (cholesterol content, 33.1%) 100 parts of pure cholesterol.

[c] 180.6 parts by weight of wool wax alcohols (triterpene alcohol content, 23.8%), 111.8 parts of pure lanosterol.

[d] 150 parts by weight of hexadecanol, 150 parts of octadecanol, 500 parts of cholesterol, 200 parts of lanosterol.

Table III. Effect of Cholesteryl Esters on Analysis for Cholesterol and Lanosterol by Method I

Sample No.[a]	Composition, % Cholesteryl palmitate	Free cholesterol	Total cholesterol	Lanosterol	Found, % Cholesterol	Found, % Lanosterol
7	1.0	27.6	28.2	19.3	29.1	19.8
8	2.0	27.3	28.6	19.1	29.7	20.3
9	4.8	26.5	29.5	18.5	30.8	19.4
10	9.1	25.1	31.2	17.6	33.6	18.0
11	16.7	22.3	34.7	15.6	38.0	15.8
12	33.3	14.0	44.9	9.9	51.5	9.3

[a] Test mixtures were prepared by adding 1, 2, 5, 10, 20, and 50 parts of cholesteryl palmitate, respectively, to six samples of a mixture consisting of 27.9 parts of cholesterol, 19.5 parts of lanosterol, and 52.6 parts of octadecanol.

or longer. The solution is cooled and neutralized by dropwise addition of 10% aqueous hydrochloric acid. A 2-ml. portion of a 1% solution of digitonin in 80% aqueous ethanol is then added, and the sample is boiled gently. Approximately 6 ml. of water is added, and the sample is again mixed and boiled for 2 to 3 minutes. It is then cooled and held at 20° C. for at least 2 hours, or preferably overnight, to allow complete precipitation of the cholesterol digitonide.

The tube is then centrifuged at 2400 r.p.m. for 5 minutes, and the supernatant liquid is carefully drawn off with a fine capillary pipet or decanted from the precipitate which is washed with 5 ml. of a mixture of equal parts of acetone and ether, using a fine, corrosion-resistant wire as a stirring rod. The tube is again centrifuged, decanted, and the same procedure is repeated twice with 5 ml. portions of ether. Since any residual ether may later cause bumping, the last traces should be removed by warming the tube to about 50° C. in a water bath and applying vacuum or a gentle stream of air.

The precipitate is dissolved in the tube by gentle warming with 12.5 ml. of glacial acetic acid, and the solution is washed into a volumetric flask and made up to exactly 100 ml. at 25° C. with chloroform. Using the modified reagent, samples are prepared and read in the spectrophotometer at 630 mμ as described for Method I. A blank consisting of 10 ml. of the acetic acid and chloroform solvent and 5 ml. of the reagent is carried through the determination. No digitonin is included; while digitonin produces no measurable color within 40 minutes, it does develop color subsequently, and a fresh blank would be required for each determination.

CALCULATION OF RESULTS

Method I. Free Alcohols.

$$\text{Cholesterol, weight \%} = \frac{100(a_{630} - a_{L'})}{a_C - a_{L'}}$$

$$\text{Triterpene alcohols, weight \%} = \frac{100(a_{550} - a_{C'})}{a_L - a_{C'}}$$

Method II. Esters.

$$\text{Cholesterol, weight \%} = \frac{100(a_{630} - a_{L'})}{1.2a_C - a_{L'}}$$

$$\text{Triterpene alcohols, weight \%} = \frac{100(a_{550} - 1.2a_{C'})}{a_L - 1.2a_{C'}}$$

Method III. Cholesterol Digitonide.

$$\text{Cholesterol, weight \%} = \frac{a_{630}}{a_{CD}} 100$$

where

a_{630} (or a_{550}) = observed absorptivity of sample

$$= \frac{\text{absorbance at 630 m}\mu\text{ (or 550 m}\mu\text{)}}{\text{(total concentration, grams/liter)(layer thickness, cm.)}}$$

a_C = absorptivity of the pure cholesterol color complex at 630 mμ = 5.00

$a_{C'}$ = absorptivity of the pure cholesterol color complex at 550 mμ = 1.50

a_L = absorptivity of the pure lanosterol color complex at 550 mμ = 5.50

$a_{L'}$ = absorptivity of the pure lanosterol color complex at 630 mμ = 0.46

a_{CD} = absorptivity of the pure cholesterol color complex equivalent of cholesterol digitonide at 630 mμ = 4.79

RESULTS

Tests of Method I were made on various mixtures of pure cholesterol and lanosterol with other alcohols as described in Table II. The results indicate a satisfactory degree of accuracy for the triterpene alcohols, especially for those mixtures which resemble most closely the material ordinarily analyzed, and a high degree of accuracy for cholesterol in all types of mixtures. In Table III it is shown that when esters of cholesterol are present, as would be the case in an incompletely saponified wool wax sample, high results are obtained, and this error is proportional to the ester content.

Tests of Method II, which is designed to be applied directly to esters of cholesterol, the triterpenes, and other alcohols without saponification, were made upon two typical wool waxes and a mixture of cholesteryl palmitate with a cholesterol-rich wool wax alcohol fraction from which most of the triterpene alcohols had been removed. The results on the wool wax samples agreed very well with those obtained from their alcohol fractions, and recovery of the added cholesterol in the synthetic sample (No. 15) was very good (Table IV). This method gives a quick and easy approximation of the cholesterol and triterpene alcohol content of wool grease although it is not as accurate as Method I.

Tests of Method III were made by comparing the results obtained by weighing the cholesterol digitonide precipitate with the cholesterol content determined from this precipitate by the absorptiometric method. As can be seen in Table V, the values agree very closely. This method would be especially useful for distinguishing between free and combined cholesterol and also for determining dihydrocholesterol if any is present.

Table IV. Analysis of Esters for Cholesterol and Lanosterol by Method II

Sample No.	Composition, %[a] Cholesterol	Composition, %[a] Lanosterol	Found, % Cholesterol	Found, % Lanosterol
13[b]	15.6	9.2	16.3	11.0
14[c]	16.9	13.2	16.1	13.3
15[d]	41.7	1.5	44.7	1.7

[a] Determined by saponifying the original esters, isolating the alcohols, and analyzing them absorptiometrically and calculating the result on the basis of the weight of the original esters.
[b] Commercial wool grease recovered by extraction from the raw wool with a petroleum solvent.
[c] Commercial wool grease recovered by centrifuging aqueous wool-scouring liquors.
[d] Mixture obtained by adding 48.7 parts of cholesteryl palmitate to 100 parts of a wool wax alcohol fraction from which the acyclic alcohols had been removed by precipitation with urea and from which most of the lanosterol had been separated by fractional precipitation of a methyl isobutyl ketone solution with methyl alcohol.

Table V. Comparison of Method III with the Gravimetric Determination of Cholesterol

Sample No.	Description	Gravimetric Determination, %	Absorptiometric Method III, %
16	Cholesterol digitonide	24.2	24.0
17	Wool wax alcohols	34.4	34.5
18	Alcohol-ester mixture A[a]	28.5	28.1
19	Alcohol-ester mixture B[a]	29.0	28.0
20	Alcohol-ester mixture C[a]	30.5	30.9
21	Alcohol-ester mixture D[a]	32.8	32.1

[a] Alcohol-ester mixtures A, B, C, and D were prepared by adding 8, 16, 40, and 80 parts of cholesteryl palmitate, respectively, to four samples of a mixture consisting of 700 parts of cholesterol, 500 parts of lanosterol, and 1300 parts of octadecanol.

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Correction.

The equations given under "Calculation of Results" for Methods I and II are incorrect. The correct equations are as follows.

Method I. Free Alcohols

$$\text{Cholesterol, weight \%} = \frac{100(a_{630}a_L - a_{550}a_{L'})}{a_C a_L - a_{C'}a_{L'}}$$

$$\text{Triterpene alcohols, weight \%} = \frac{100(a_{550}a_C - a_{630}a_{C'})}{a_C a_L - a_{C'}a_{L'}}$$

Method II. Esters.

$$\text{Cholesterol, weight \%} = \frac{100(a_{630}a_L - a_{550}a_{L'})}{1.2a_C a_L - 1.2a_{C'}a_{L'}}$$

$$\text{Triterpene alcohols, weight \%} = \frac{100(a_{550}a_C - a_{630}a_{C'})}{a_C a_L - a_{C'}a_{L'}}$$